Supplemental Data

KLOTHO INHIBITS TGF-β1 SIGNALING AND SUPPRESSES RENAL FIBROSIS AND CANCER METASTASIS IN MICE

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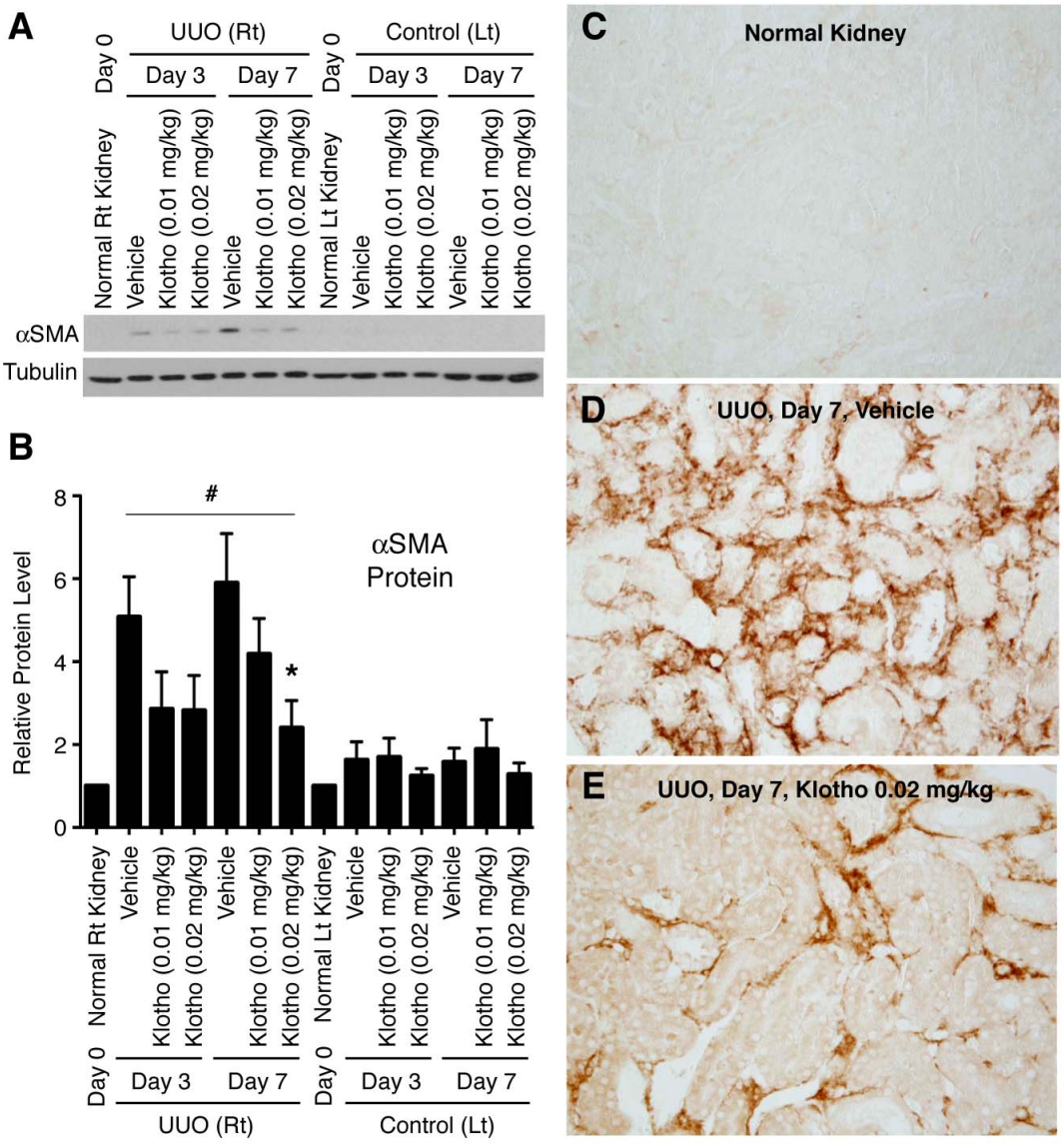
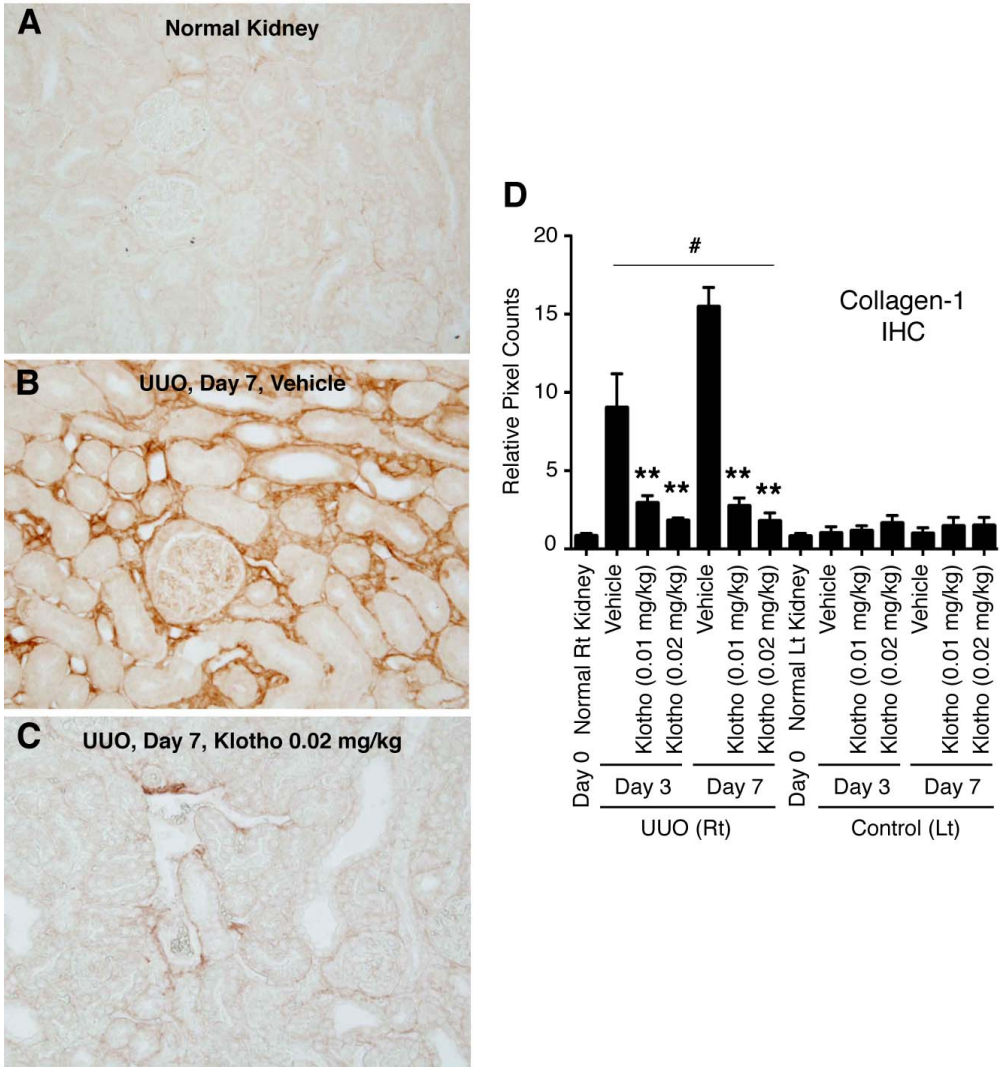
This Supplemental Data includes:

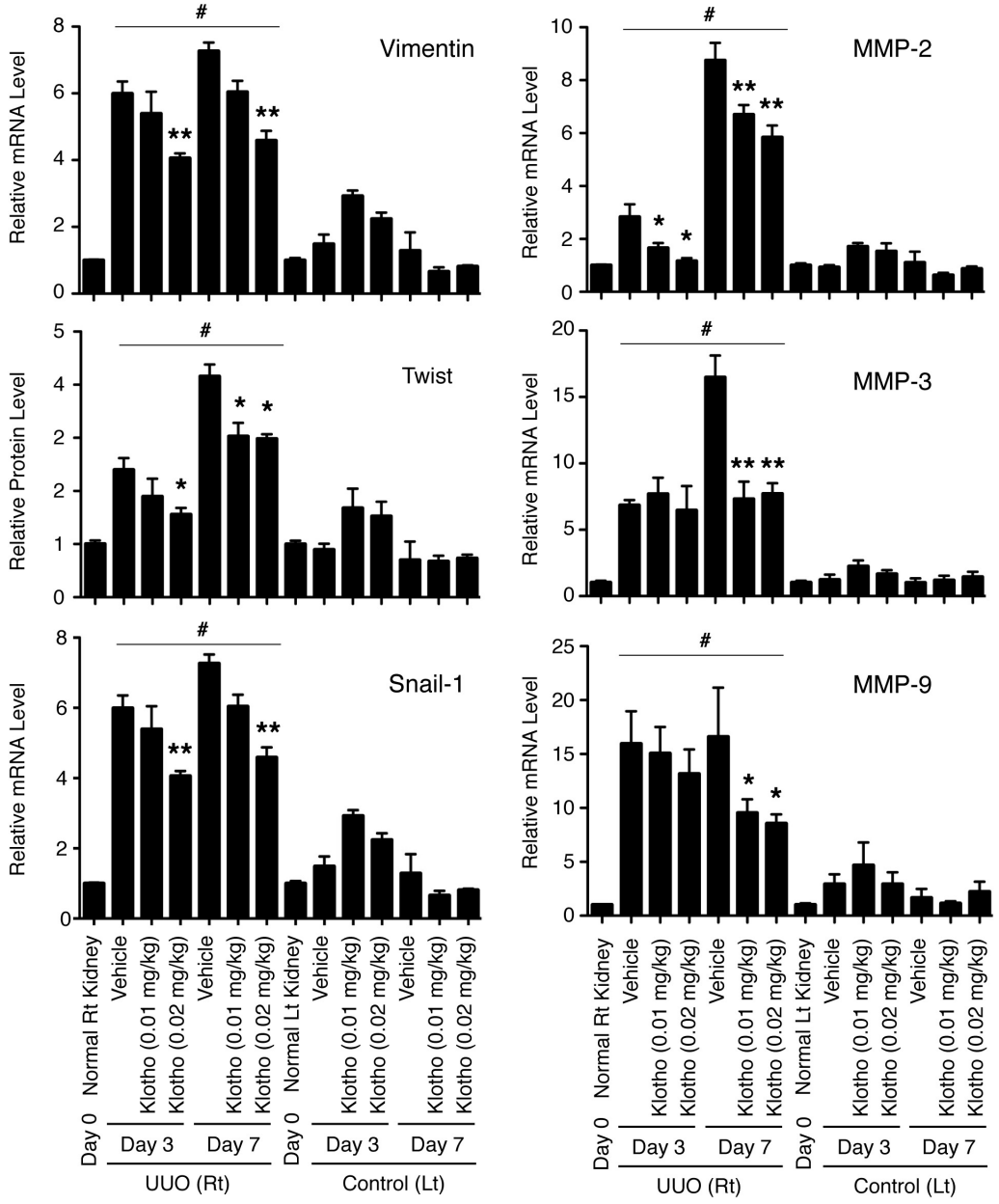
- Supplemental Figure 1-6
- Supplemental Table 1
- Supplemental references



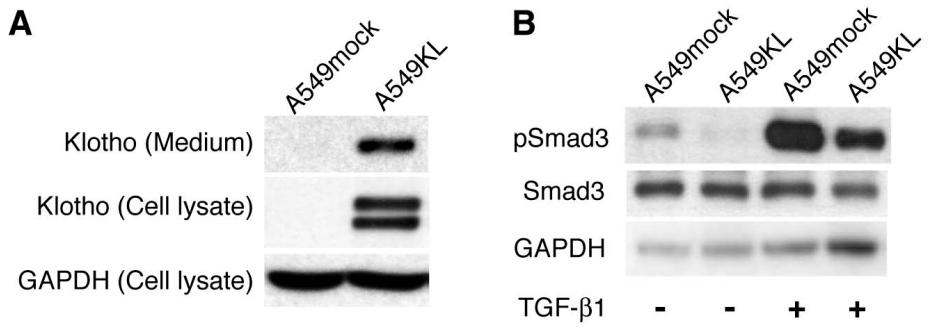
Supplemental Figure 1 Klotho protein injection suppresses increased α-smooth muscle actin protein induced by UUO. (**A**) Typical western blot of α-smooth muscle actin (αSMA). Lysates of right (UUO) and left (Control) kidneys from mice treated with vehicle or Klotho (0.01 mg/kg or 0.02 mg/kg) for 0 (Normal), 3 or 7 days were subjected to immunoblot analyses using antibodies against αSMA and tubulin. (**B**) Quantification of signal intensity of αSMA in immunoblot analyses. The αSMA/tubulin ratios were normalized with those of normal kidney. Data indicate means ± SEM (n = 5 per treatment & time point). $^{*}P < 0.05$ vs vehicle-treated mice at the same time point by two-tailed t test. $^{\#}P < 0.05$ vs mice at Day 0 by two-tailed t test. (**C-E**) Typical immunohistochemistry of αSMA in normal kidney (**C**) and in UUO kidney treated with vehicle (**D**) or Klotho (0.02 mg/kg) (**E**) for 7 days. Paraffin sections of kidneys were stained with anti-αSMA antibody as described (1).



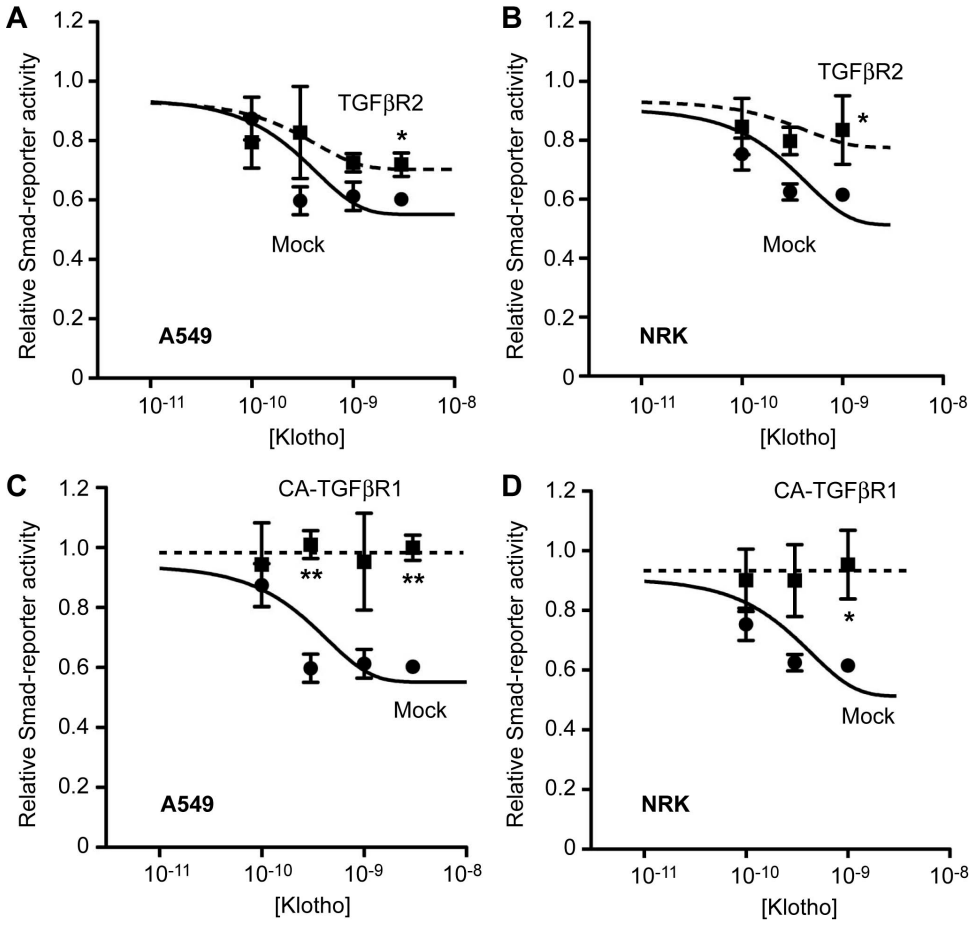
Supplemental Figure 2 Klotho protein injection suppresses increased Collagen-1 protein induced by UUO. (**A-C**) Typical immunohistochemistry of Collagen-1 in normal kidney (**A**) and in UUO kidney treated with vehicle (**B**) or Klotho (0.02 mg/kg) (**C**) for 7 days. Paraffin sections of kidneys were stained with anti-Collagen-1 antibody as previously described (1). (**D**) Quantification of signal intensity of Collagen-1 in immunohistochemistry. Five fields under a high power field (original magnification x400) were randomly selected from sections of right (UUO) and left (Control) kidneys from mice treated with vehicle or Klotho for 0 (Normal), 3 or 7 days. Signal intensities were quantified using ImageJ software. Pixel counts were normalized with those of normal kidney. Data indicate means ± SEM (n = 5 per treatment & time point). $^{**}P < 0.01$ vs vehicle-treated mice at the same time point by two-tailed t test. $^{\#}P < 0.05$ vs mice at Day 0 by two-tailed t test.



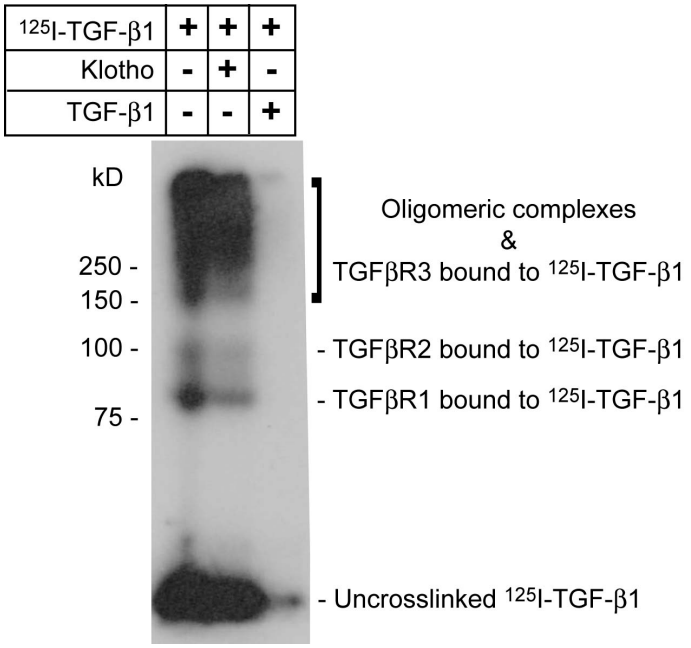
Supplemental Figure 3 Klotho protein injection suppresses increased mesenchymal marker expression induced by UUO. RNA of right (UUO) and left (Control) kidneys from mice treated with vehicle or Klotho (0.01 mg/kg or 0.02 mg/kg) for 0 (Normal), 3 or 7 days were subjected to qPCR to quantify mRNA levels of Vimentin, Twist, Snail-1, and metalloproteases (MMP-2, -3, and -9). The mRNA level was normalized with that of normal kidney. Data indicate means ± SEM (n = 5 per treatment & time point). *$P < 0.05$, **$P < 0.01$ vs vehicle-treated mice at the same time point by two-tailed t test. $^{\#}P < 0.05$ vs mice at Day 0 by two-tailed t test.



Supplemental Figure 4 Forced expression of Klotho attenuates TGF-β1 signaling in A549 cells. (**A**) A549 cell lines were established by stable transfection of the Klotho expression vector (A549KL) or empty vector (A549mock) as described in Methods. Cell lysate and conditioned medium were subjected to immunoblot analysis using anti-Klotho antibody (KM2119). A549KL cells released Klotho ectodomain into the conditioned medium. Klotho protein in cell lysate was detected in doublets in western blot. The upper and lower bands represent fully-glycosylated and underglycosylated Klotho protein, respectively (2). (**B**) A549KL and A549mock cells were stimulated with TGF-β1 (0.25 ng/ml) or left untreated for 30 minutes. Cell lysates were subjected to immunoblot analysis using antibody against phosphorylated Smad3 (pSmad3), antibody that recognized Smad3 regardless of its phosphorylation state (Smad3), or anti-GAPDH antibody.



Supplemental Figure 5 The point of Klotho action is TGFβR2. (**A, B**) Over-expression of TGFβR2 attenuated the ability of Klotho to inhibit TGF-β1 signaling. A549 cells (**A**) and NRK52E cells (**B**) were transfected with mock or expression vectors for TGFβR2 together with the Smad-sensitive reporter and LacZ expression vector for the reporter assay. Sixteen (16) hours after transfection, cells were stimulated with TGF-β1 (2 ng/ml) and Klotho (0 - 3 nM). Twenty-four (24) hours later, cell lysates were subjected to measurement of luciferase and β-galactosidase activity. Data indicates relative Smad-reporter activity after normalization with β-galactosidase activity (n = 3 for each data point). Closed circles with solid inhibition curve represent mock-transfected cells. Closed squares with dotted inhibition curve represent TGFβR2-transfected cells. (**C, D**) Over-expression of constitutively active TGFβR1 abolished the ability of Klotho to inhibit TGF-β1 signaling. As in **A** and **B**, except that constitutively active TGFβR1 expression vector was used instead of TGFβR2 expression vector. **P* < 0.05, ***P* < 0.01 vs mock by two-tailed t-test.



Supplemental Figure 6 Klotho inhibits TGF-β1 binding to TGFβR2. Cross-linking of ^{125}I- TGF-β1 to cell surface receptors was performed as described(3). Briefly, subconfluent A549 cells on 10 cm dishes were incubated with ^{125}I- TGF-β1 (0.25 nM), Klotho (10 nM), or TGF-β1 (25 nM) in binding buffer (KRH with 0.1% BSA) for 60 minutes at room temperature. After washing with PBS, water-soluble, non-cleavable cross-linker (BS^3, Thermo Scientific) was added at 0.5 mg/ml and incubated on ice for 30 minutes. After quenching the cross-linking reaction by Tris (10 mM), cells were washed with PBS, lysed, and subjected to SDS-PAGE under reducing condition. Cell-surface protein cross-linked to ^{125}I-TGF-β1 was detected by autoradiography. Identity of the bands was based on the apparent molecular weight.

Supplemental Table 1 Primers used for qPCR.

mRNA	Forward primer	Reverse primer
Klotho	AATTATGTGAATGAGGCTCTGAAAG	TACGCAAAGTAGCCACAAAGG
αSMA	CTGACAGAGGCACCACTGAA	CATCTCCAGAGTCCAGCACA
Collagen-1	GAGCGGAGAGTACTGGATCG	GTTCGGGCTGATGTACCAGT
TGF-β1	TTGCTTCAGCTCCACAGAGA	TGGTTGTAGAGGGCAAGGAC
Vimentin	CTGCACGATGAAGAGATCCA	AGCCACGCTTTCATACTGCT
MMP-2	ACCCTGGGAGAAGGACAAGT	ATCACTGCGACCAGTGTCTG
MMP-3	CAGACTTGTCCCGTTTCCAT	GGTGCTGACTGCATCAAAGA
MMP-9	CAATCCTTGCAATGTGGATG	AGTAAGGAAGGGGCCCTGTA
Snail-1	CTTGTGTCTGCACGACCTGT	CTTCACATCCGAGTGGGTTT
Twist	CTCGGACAAGCTGAGCAAG	CAGCTTGCCATCTTGGAGTC
Cyclophilin	TGGAGAGCACCAAGACAGACA	TGCCGGAGTCGACAATGAT

Supplemental references

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2. Imura, A., Iwano, A., Tohyama, O., Tsuji, Y., Nozaki, K., Hashimoto, N., Fujimori, T., and Nabeshima, Y. (2004) *FEBS Lett* **565**, 143-147
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